bio-protocol Preprint

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Content mixing assay

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An abbreviated version of this protocol was published in eLIFE in Apr 2016

Synaptotagmin-1 C2B domain interacts simultaneously with SNAREs and membranes to promote membrane fusion

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Detailed protocol

1. **Reagents setup**

i) lipids:

POPC (10 mg/ml, Avanti Polar Lipids #850457)
POPE (10 mg/ml, Avanti Polar Lipids #850757)
DOPS (10 mg/ml, Avanti Polar Lipids #840035)
PI-4,5-P_2 (1 mg/ml, Avanti Polar Lipids #840046)
Cholesterol (Chol, 10 mM, Avanti Polar Lipids #700000)
***All lipids were dissolved in chloroform except for PI-4,5-P2 in chloroform:methanol:water = 20:9:1**

ii) Chemicals:

Tris(2-carboxyethyl)phosphine (TCEP, Sigma Aldrich #C4706), dissolved in deionized water at 0.5 M
3-[(3-Cholamidopropyl)dimethylammonio]-1-propane sulfate (CHAPS, Amresco [now avantor VWR #VWRV0465]), dissolved in deionized water at 20% (w/v)
Sulforhodamine B (SRB, Sigma Aldrich #230162), dissolved in lipid dissolving buffer (see below)
Bio-Beads SM2 (Bio-Rad #1523920)
poly-D-lysine hydrobromide (mol wt 30,000-70,000, Sigma Aldrich #P7886), dissolved in deionized water at 200 ug/ml

iii) Buffers:

Lipid dissolving buffer I: 25mM HEPES-K pH 7.40, 150 mM KCl, 10% (v/v) Glycerol, 0.2 mM TCEP, 1% (w/v) CHAPS
Lipid dissolving buffer II: 25mM HEPES-K pH 7.40, 150 mM KCl, 10% (v/v) Glycerol, 0.2 mM TCEP, 50 mM SRB, 1% (w/v) CHAPS
Dialysis buffer I: 5mM HEPES-K pH 7.40, 150 mM KCl, 10% (v/v) Glycerol, 0.1 mM TCEP
Dialysis buffer II: 5mM HEPES-K pH 7.40, 150 mM KCl, 10% (v/v) Glycerol, 0.1 mM TCEP, 40 mM SRB
Reaction buffer: 5mM HEPES-K pH 7.40, 150 mM KCl, 10% (v/v) Glycerol, 0.1 mM TCEP
$CaCl_2$ solution: 0.1 M, dissolved in deionized water
$EDTA\text{-}2Na^+$ solution: 50 mM, dissolved in deionized water

2. Instruments and consumables

QM40 spectrofluorimeter (Photo Technology International, now part of Horiba)
Vortex-Genie 2 (Scientific industries)
Magstir Genie (Scientific industries)
Oil pump (Oerlikon Leybold Vacuum)
Vacuum chamber (Shanghai Everone)
Flasks 250ml, 1L (Fisher)
Glass test tube Φ8mm (Customization)
Slide-A-Lyzer™ Dialysis Cassette (3.5K MWCO, 0.5 mL, Thermo Scientific #66335)

3. Experiments

i) Proteoliposome preparation

Lipids formula (for prepare 200 ul, 5mM total lipids):

Target liposome (no SRB loading)			*Donor liposome (SRB loading)*		
Lipids	*mol%*	*volume*	*Lipids*	*mol%*	*volume*
POPC	*59%*	*45.8ul*	*POPC*	*55%*	*42.7ul*
POPE	*20%*	*14.0ul*	*POPE*	*20%*	*14.0ul*
DOPS	*20%*	*16.0ul*	*DOPS*	*15%*	*12.0ul*
PI-4,5-P_2	*1%*	*11.0ul*	*Chol*	*10%*	*10.0ul*

Lipids were mixed in glass test tubes, dry under nitrogen flow with gentle rotation.
*** Important: For lipid mixtures containing PI-4,5-P_2, one should use 45°C water bath when drying under nitrogen flow. This is because of the**

Important: For lipid mixtures containing PI-4,5-P_2, one should use 40°C water bath when drying under nitrogen flow. This is because of the higher transition temperature (~40°C) of currently used PI-4,5-P_2.

After drying under nitrogen flow (until no visible liquids, one could easily see lipid films stick on the glass tube), the glass test tubes were transferred to a vacuum chamber equipped with an oil pump, keep the lipid films under vacuum for at least 3 hours (room temperature).

Then dissolve the lipid films using the formula as follow:

Target liposome (no SRB loading)		*Donor liposome (SRB loading)*	
Lipid dissolving buffer I	*160ul*	*Lipid dissolving buffer II (containing SRB)*	*165ul*
Syx1/SN25 complex(125 uM)	*40ul*	*Syb2(200 uM)*	*25ul*
		Syt1(100 uM)	*10ul*

The protein-to-lipid (p/l) ratio is 1:200 for Syx1/SN25 complex and Syb2, 1:1,000 for Syt1. Vortex the mixtures on ice for 5 minutes until the lipid film fully dissolved, then incubate the mixture at room temperature for 30 minutes.

*** It is recommended to incubate the dissolved lipid films at room temperature for better performance (transmembrane protein incorporation).**

Transfer the mixtures to Slide-A-Lyzer™ Dialysis Cassette, for SRB-loaded donor liposome, firstly dialyze in 250 ml Dialysis buffer II supplied with 1.25 g Bio-Beads SM2 (5g/L) at 4°C in the dark for 6 hours.

*** Important: this step aims to keep constant 40 mM SRB loaded into donor proteoliposome.**

For the following dialysis, target liposomes and SRB-loaded donor liposomes are the same: using 1L Dialysis buffer I (no SRB supplied) supplied with 5g Bio-Beads SM2 (5g/l). Dialyze 3 times at 4°C in the dark, 6 hours for each time.

*** Prepared proteoliposomes could be stored at 4°C in the dark no more than 3 days for better performance.**

ii) Content mixing assay

Reaction setup: (60ul cuvette)

Donor liposome (5 mM)	*1.2 ul (100 uM)*
Target liposome (5 mM)	*0.6 ul (50 uM)*
EDTA-2Na^+(50 mM)	*0.24 ul (0.2 mM)*
Complexin-1 (200 uM)	*6.0 ul (20 uM)*
poly-D-lysine (200 ug/ul)	*0.6 ul (2 ug/ml)*
Reaction buffer	*51.4 ul*

Instrument setup:

Excitation wave-length	*565 nm*
Emission wave-length	*580 nm*
Slit width (excitation/emission)	*1.25 mm/1.25 mm*
Temperature	*25 °C*
Sampling rate	*1 Hz*

During the sampling at 1,500 s, supply 0.6 ul $CaCl_2$ solution (the final concentration is 1 mM) to trigger fast liposome fusion.

How to cite:(Readers should cite both the Bio-protocol preprint and the original research article where this protocol was used)

1. Wang, S. (2020). Content mixing assay. Bio-protocol Preprint. bio-protocol.org/prep352.
2. Wang, S., Li, Y. and Ma, C.(2016). Synaptotagmin-1 C2B domain interacts simultaneously with SNAREs and membranes to promote membrane fusion. eLIFE. DOI: 10.7554/eLife.14211